A Model for Prediction of Heat Stability of Photosynthetic Membranes

Zoran Ristic,* Urška Bukovnik, P. V. Vara Prasad, and Mark West

ABSTRACT

A previous study revealed a positive correlation between heat-induced damage to photosynthetic membranes (thylakoid membranes) and chlorophyll loss. In this study, we exploited this correlation and developed a model for predicting thermal damage to thylakoids. Prediction is based on estimation of the ratio of constant chlorophyll *a* fluorescence (O) and the peak of variable fluorescence (P) (O/P). The model was developed using 12 cultivars of hexaploid winter wheat (*Triticum aestivum* L.). It was tested in six genotypes of hexaploid wheat, 25 genotypes of tetraploid wheat (*T. turgidum* L.), and 20 genotypes of maize (*Zea mays* L.). Predictive ability was assessed by analyzing the relationship between the predicted and measured O/P. The model adequately predicted O/P and thereby the heat stability of thylakoid membranes in all genotype groups with high coefficients of determination ($r^2 > 0.80$). This model could be used as an easy and inexpensive means for detection of the structural and functional state of photosynthetic membranes in wheat and maize, and possibly other crops, in hot environments.

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Abbreviations: O, constant chlorophyll *a* fluorescence; P, peak of variable fluorescence; PPF, photosynthetic photon flux; PSII; Photosystem II; RMSPR, prediction root mean square error.

Chloroplast photosynthetic membranes, thylakoids, are considered the most heat labile cell structures (Santarius, 1974; Schreiber and Berry, 1977). In many crop species, they are more affected by heat stress than the chloroplast envelope, stromal enzymes, or the integrity of cell compartments (Thebud and Santarius, 1982; Monson et al., 1982; Kobza and Edwards, 1987; Sayed et al., 1989; Al-Khatib and Paulsen, 1989). The negative effects of heat stress on thylakoid membranes are manifested by thylakoid swelling (Ristic and Cass, 1991, 1992) and increased leakiness (Bukhov et al., 1999; Schrader et al., 2004), physical separation of the chlorophyll light-harvesting complex II (LHCII) from the Photosystem II (PSII) core complex (Schreiber and Berry, 1977; Armond et al., 1980; Pastenes and Horton, 1999), and disruption of the PSII-mediated electron transfer (Berry and Björkman, 1980; Sharkey, 2005). The structural and functional damage of thylakoids is thought to be a major constraint of photosynthesis and plant productivity in hot environments (Berry and Björkman, 1980).

Determination of damage to thylakoid membranes is often used as a reliable means of assessing the plant's susceptibility to heat stress (Krause and Weis, 1984; Ristic and Cass, 1993; Ristic

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et al., 1998; 2008). Damage to thylakoid membranes can be estimated by measuring chlorophyll *a* fluorescence and determining the ratio of constant fluorescence (O) and the peak of variable fluorescence (P) (Krause and Weis, 1984; Ristic and Cass, 1993; Ristic et al., 1998, 2008). Increase in the O/P ratio indicates damage to thylakoid membranes; the higher the increase the greater the damage (Krause and Weis, 1984; Ristic et al., 1998, 2008).

Although chlorophyll fluorescence provides a reliable means of determining the heat stability of thylakoid membranes, this technique has some limitations. Measurements of chlorophyll *a* fluorescence require expensive instrumentation and in some cases necessitate dark adaptation of the leaf tissue, which limits the number of plants that can be screened on a given day. Therefore, alternative approaches for quick, reliable, and inexpensive assessment of thylakoid heat stability are needed. A possible approach for such assessment would include a model that could predict the stability of thylakoid membranes in hot environments.

We recently investigated the heat stability of thylakoid membranes and chlorophyll content in cultivars of winter wheat (*Triticum aestivum* L.) under heat stress conditions (Ristic et al., 2007). The heat stability of thylakoid membranes was assessed using chlorophyll *a* fluorescence and chlorophyll content was measured with a SPAD chlorophyll meter. The results showed that heat stress caused damage to thylakoids and loss of chlorophyll. The results also showed a strong negative linear correlation between the heat damage to thylakoid membranes and chlorophyll content ($p < 0.0001$).

In this study, we exploited the relationship between chlorophyll content and thylakoid heat stability (Ristic et al., 2007) and tested the hypothesis that chlorophyll content, as determined by a SPAD chlorophyll meter, can predict thermal damage to thylakoid membranes. We developed a model for prediction of thermal damage to thylakoids and tested this model in both wheat and maize (*Zea mays* L.).

MATERIALS AND METHODS

Model Development

Plant Material, Growth Conditions, and Heat Treatment

Twelve cultivars of hexaploid winter wheat with varying tolerance to heat stress (Ristic et al., 2008) were used to develop a prediction model of thermal damage to thylakoid membranes. Five of the cultivars (Zlatka, Stepa, NS2-4523, Rana Niska, and Kompas) were previously classified as heat susceptible, and seven (Proteinka, Ljiljana, Partizanka, NS2-4992, Dragana, Stamena, and Jefimija) were classified as heat tolerant (Ristic et al., 2008). Two experiments were conducted in the laboratory. Plant growth conditions and heat treatment were similar to those described by Ristic et al. (2007). Briefly, plants of each cultivar were grown in 10 pots (Metro Mix 200 potting soil [Hummert Intl., Topeka, KS], three seedlings per pot) in a greenhouse and were watered daily and fertilized weekly for the entire duration of the experiment (Ristic et al., 2007). At the beginning of flowering stage (50% of the plants at growth stage Feekes 10.5.1 [Large, 1954]), plants of each cultivar were divided into control (five pots) and heat treatment (five pots) groups. In each group, five plants were randomly selected (one plant per pot), and one flag leaf per selected plant was randomly chosen and tagged (total of five flag leaves per group were tagged). The tagged leaves were later used for measurements of chlorophyll *a* fluorescence and chlorophyll content. The treatment group was exposed to heat stress for 16 d (day/night temperature, 36/30°C; relative humidity, 90–100%; photoperiod, 16/8 h; photosynthetic photon flux [PPF], 280 μmol m^{-2} s^{-1} [Sylvania cool white fluorescent lamps]) in a growth chamber (Conviron, Model PGW-36, Winnipeg, MB, Canada), and the control group was maintained under growth conditions in a greenhouse (average daily temperature in the greenhouse was 22.7 ± 2.8°C; during the period of heat stress treatment, the PPF in the greenhouse ranged from 270 to 320 μmol m^{-2} s^{-1} [photoperiod, 16/8 h; supplemental light was used to extend daylight period]). For each cultivar, heat treatment started when 50% of the plants reached growth stage Feekes 10.5.1 (Large, 1954).

To avoid or minimize possible dehydration of the leaf tissue during stress treatment, pots of the treatment and control group were kept in trays containing ~1 cm deep water. Chlorophyll *a* fluorescence and chlorophyll content were measured after 0, 2, 4, 6, 8, 10, 12, 14, and 16 d of heat stress. Chlorophyll *a* fluorescence and chlorophyll content were measured on the same intact flag leaves as described by Ristic et al. (2007). A self-calibrating SPAD chlorophyll meter (Model 502, Spectrum Technologies, Plainfield, IL) was used for chlorophyll measurements. Data from five replicate plants were averaged at each day of heat stress, and averages of O/P and chlorophyll content from the 12 cultivars from two separate experiments were used for model development. We refer to this data as the *development data*.

Prediction Model

A model for prediction of thermal damage to thylakoid membranes was developed using two approaches. In the first approach, Model C, chlorophyll content in heat stressed plants was expressed as a percentage of the chlorophyll content in control plants (predictor variable *x*). This percentage was fit to the O/P ratio of chlorophyll *a* fluorescence (response variable *y*) using random coefficients linear regression (Laird and Ware, 1982). Random coefficients regression was used to account for variation among slopes and intercepts among the genotypes used for model development. The form of the model fit was

$$y_{ijk} = \beta_0 + E_i + b_{0j} + (\beta_1 + b_{1j})x_k + e_{ijk}$$

where index *i* (2 levels) represents the *i*th experiment, index *j* (12 levels) represents the *j*th level of genotype, and index *k* (9 levels) represents the *k*th level of chlorophyll content measured on the *i*th species. The terms β_0 and β_1 in the model represent the fixed effects intercept and slope, respectively, whereas the terms b_{0j} and b_{1j} represent random intercepts and slopes associated with genotypes, in that order. The E_i represents errors associated with experiments, and the e_{ijk} represents remaining unexplained residual error for the *ijk*th observation. The usual

assumptions of normality and homoscedasticity were made for residual errors. Multivariate normality with zero means and no particular form for covariance was assumed for the random regression coefficients b_{0j} and b_{1j}. The random effects of experiment E_i were assumed to be normal with zero mean. Only the estimates of the fixed effects, $\hat{\beta}_0$ and $\hat{\beta}_1$, were used for prediction of O/P ratio to give a simple linear regression prediction model of the form $\hat{y} = \hat{\beta}_0 + \hat{\beta}_1 x$. The nlme package in R (R Development Core Team, 2007) was used to fit the random coefficients models. In the second approach, the model (Model-HS) was developed in the same way as in the first approach, with the exception that the chlorophyll content in heat-stressed plants was expressed as a percentage of the chlorophyll content in the same plants at the beginning of heat treatment (Day 0 of heat stress).

Model Testing

Plant Material, Growth Conditions, and Heat Treatment

Three genotypes of hexaploid winter wheat, 3 genotypes of hexaploid spring wheat, 25 genotypes of tetraploid wheat (*T. turgidum* L.) (Table 1), and 20 genotypes of maize (Table 2) were used to test the model for prediction of thermal damage to thylakoid membranes. Data for model testing in wheat and maize were generated in separate experiments. For testing in wheat, plants of each genotype were grown in six pots (three plants per pot) in a greenhouse and were watered and fertilized as described in "Model Development" above. At early flowering stage (growth stage Feekes 10.5.1. [Large, 1954]), plants were divided into control (three pots) and treatment–heat-stress (three pots) groups. The control group was maintained under

Table 1. Comparison between predicted and measured ratio of constant chlorophyll *a* fluorescence (O) and the peak of variable fluorescence (P) (O/P) in wheat.

Triticum species	Cultivar/ subspecies	Wheat type/ICARDA accession no.	Geographical origin	Model C			Model HS			Change in chlorophyll content‡
				r^2	RMSPR†	*d* value	r^2	RMSPR	*d* value	
T. aestivum	Reska	Winter wheat	Slovenia	0.0044	0.0290	0.2962	0.0270	0.0181	0.4529	ns
T. aestivum	Gorolka	Winter wheat	Slovenia	0.1380	0.0548	0.2828	0.0179	0.0189	0.2610	ns
T. aestivum	Ventnor	Winter wheat	Australia	0.9449	0.0818§	0.8691	0.9105	0.0840§	0.8650	$p < 0.01$
T. aestivum	Kukri	Spring wheat	Australia	0.8288	0.0820§	0.9332	0.8333	0.7892§	0.9428	$p < 0.01$
T. aestivum	RAC-875	Spring wheat	Australia	0.9074	0.0634	0.9556	0.9006	0.5955	0.9633	$p < 0.01$
T. aestivum	Excalibur	Spring wheat	Australia	0.9650	0.0622	0.9354	0.9703	0.0579	0.9476	$p < 0.01$
T. turgidum	*dicoccon*	45067	Oman	0.0647	0.0340	0.3734	0.4228	0.0261	0.6795	ns
T. turgidum	*dicoccon*	45303	Ethiopia	0.0453	0.0461	0.1874	0.2003	0.0353	0.0958	ns
T. turgidum	*dicoccon*	45304	India	0.0152	0.0695	0.1588	0.0161	0.0736	0.2134	ns
T. turgidum	*dicoccon*	45363	Palestine	0.7239	0.0523	0.9130	0.6185	0.0704	0.8493	$p < 0.01$
T. turgidum	*dicoccon*	45393	Eritrea	0.0843	0.0958§	0.1670	0.0675	0.0587	0.0963	ns
T. turgidum	*dicoccon*	88750	Afghanistan	0.9710	0.0378	0.9705	0.9476	0.0292	0.9827	$p < 0.01$
T. turgidum	*dicoccon*	45069	Oman	0.8261	0.0736	0.9310	0.8969	0.0499	0.9677	$p < 0.01$
T. turgidum	*dicoccon*	45388	Georgia	0.9576	0.0624	0.9611	0.9490	0.0547	0.9737	$p < 0.01$
T. turgidum	*dicoccon*	45400	Iran	0.7812	0.0893§	0.7844	0.8897	0.0590	0.8875	$p < 0.01$
T. turgidum	*dicoccon*	45413	Bulgaria	0.8676	0.0774§	0.9144	0.8598	0.0698	0.9378	$p < 0.01$
T. turgidum	*dicoccon*	45416	Armenia	0.6037	0.0831	0.8802	0.6301	0.0902§	0.8806	$p < 0.01$
T. turgidum	*dicoccon*	88723	Greece	0.3068	0.0631	0.4403	0.2555	0.0685	0.3339	$p < 0.01$
T. turgidum	*dicoccon*	88763	India	0.5520	0.0228	0.7754	0.5172	0.0301	0.6605	ns
T. turgidum	*dicoccon*	99236	Yemen	0.9815	0.0265	0.9811	0.9667	0.0259	0.9834	$p < 0.01$
T. turgidum	*polonicum*	45270	Turkey	0.8366	0.0185	0.9145	0.8947	0.0192	0.9150	ns
T. turgidum	*polonicum*	97746	Ethiopia	0.7930	0.0296	0.9330	0.7545	0.0303	0.9270	$p < 0.01$
T. turgidum	*polonicum*	127682	Palestine	0.8890	0.0608	0.9253	0.9413	0.0567	0.9377	$p < 0.01$
T. turgidum	*polonicum*	110572	Algeria	0.2278	0.0550	0.5453	0.1227	0.0495	0.4920	ns
T. turgidum	*polonicum*	127684	Morocco	0.7876	0.0430	0.7496	0.4529	0.0515	0.6024	ns
T. turgidum	*turanicum*	82822	Turkey	0.5299	0.0860§	0.8082	0.5667	0.0723	0.8628	$p < 0.01$
T. turgidum	*turanicum*	83963	Afghanistan	0.2223	0.0502	0.6173	0.1707	0.0536	0.5828	ns
T. turgidum	*turanicum*	85496	Egypt	0.8295	0.0297	0.9370	0.7886	0.0391	0.9044	ns
T. turgidum	*turgidum*	45448	Ethiopia	0.9219	0.0824§	0.9363	0.9230	0.0734	0.9511	$p < 0.01$
T. turgidum	*turgidum*	83035	Turkey	0.9132	0.0579	0.9252	0.9072	0.0449	0.9597	$p < 0.01$
T. turgidum	*carthlicum*	44999	Turkey	0.9293	0.0512	0.9798	0.9228	0.0566	0.9760	$p < 0.01$

†RMSPR, prediction root mean square error.

‡Indicates change in chlorophyll content after 16 d of heat stress, as compared to the chlorophyll content in control plants (*t*-test, assuming unequal variances) or chlorophyll content in heat-stressed plants at Day 0 of heat stress (paired *t*-test); chlorophyll content in control plants and chlorophyll content in heat-stressed plants at Day 0 of heat stress were similar (not shown); ns, not significant.

§Indicates that RMSPR is significantly greater ($p < 0.05$) than RMSE based on *F* test.

Table 2. Comparison between predicted and measured ratio of constant chlorophyll *a* fluorescence (O) and the peak of variable fluorescence (P) (O/P) in maize.†

Genotype ID	Model C			Model HS		
	r^2	RMSPR‡	*d* value	r^2	RMSPR	*d* value
1	0.9461	0.0505	0.9535	0.9237	0.0601	0.9499
2	0.9512	0.0327	0.9827	0.9356	0.0369	0.9783
3	0.9704	0.0450	0.9798	0.9770	0.0343	0.9891
4	0.9166	0.0678	0.8872	0.9472	0.0470	0.9436
5	0.9677	0.0506	0.9605	0.9744	0.0455	0.9727
6	0.9532	0.0332	0.9807	0.9493	0.0323	0.9833
7	0.9508	0.0486	0.9777	0.9532	0.0451	0.9810
8	0.7157	0.1171§	0.4000	0.8279	0.0590	0.7152
9	0.9600	0.0410	0.9855	0.9644	0.0381	0.9883
10	0.9310	0.0550	0.9505	0.9468	0.0450	0.9685
11	0.9371	0.0796§	0.9280	0.9364	0.0579	0.9673
12	0.9122	0.0596	0.9165	0.9108	0.0408	0.9627
13	0.9442	0.0658	0.9548	0.9616	0.0426	0.9844
14	0.9082	0.0531	0.9698	0.9148	0.0534	0.9708
15	0.8134	0.0480	0.9474	0.8465	0.0478	0.9531
16	0.9730	0.0828§	0.9145	0.9793	0.0351	0.9871
17	0.9635	0.0688	0.9643	0.9380	0.0647	0.9681
18	0.9828	0.0406	0.9867	0.9859	0.0315	0.9931
19	0.9131	0.0904§	0.9256	0.9165	0.0697	0.9621
20	0.9118	0.0597	0.9274	0.9367	0.0540	0.9426

†For proprietary reasons, the name of maize genotypes are not revealed.

‡RMSPR, prediction root mean square error.

§Indicates that RMSPR is significantly greater ($P < 0.05$) than RMSE based on *F* test.

growth conditions in a greenhouse, and the treatment group was exposed to heat stress in a growth chamber for 16 d. The heat treatment and the nutrient, water, and light regimes were similar to those described for model development. During the exposure to heat stress, chlorophyll *a* fluorescence and chlorophyll content were measured at 2-d intervals starting at Day 0 of heat treatment. Chlorophyll *a* fluorescence and chlorophyll content were measured as outlined above, with the exception that in each group (control and treatment) three randomly chosen plants (one plant from each of three replicate pots) were used for measurements. Both O/P ratios and chlorophyll content from three replicate plants were averaged for each day of heat exposure and used for model testing.

For model testing in maize, seeds of each genotype were planted in six pots (one seed per pot; pot diam.: 15 cm top, 12 cm bottom; pot height: 17 cm) containing potting soil (Metro Mix 350; Hummert Int., Topeka, KS) and 15 g of Osmocote (slow-release fertilizer; Scotts-Sierra Horticultural Products Co., Marysville, OH). Plants were grown in a greenhouse and were watered daily during the entire duration of the experiment. Forty-five-d-old plants were divided into control (three pots) and heat stress (three pots) groups. In each group, the top fully expanded leaves of three replicate plants were tagged, and the tagged leaves were later used for measurements of chlorophyll *a* fluorescence and chlorophyll content. The control group was then transferred to a growth chamber set at 25/20°C (day/night), and the treatment group was transferred to a chamber set at 39/34°C (day/night). The relative humidity, photoperiod, and PPF in the two chambers were similar (relative humidity, 90–100%; photoperiod, 16/8 h; PPF, 280 μmol m^{-2} s^{-1} [Sylvania cool white fluorescent lamps]). Plants of heat stress group were exposed to 39/34°C (day/night) for 10 d, after which they were heated at 42/39°C (day/night) for an additional 6 d in the same chamber; the additional heating was introduced to ensure plants were affected by heat stress. To minimize or avoid possible dehydration of the leaf tissue during stress treatment, all the pots, including controls, were kept in trays containing ~1 cm deep water. During the 16-d heat treatment, chlorophyll *a* fluorescence and chlorophyll content were measured in both control and heat stressed plants at 2-d intervals as described in "Model Development." The O/P ratio was then determined (measured O/P). Both O/P ratio and chlorophyll content from three replicate plants were averaged on each day of heat exposure and used for model testing. These data are referred to as *test data* for further discussions.

Model Evaluation

Model-C and Model-HS were used to predict damage to thylakoid membranes in heat-stressed plants represented in the test data. Damage to thylakoid membranes was predicted by estimating the O/P ratio of chlorophyll *a* fluorescence (predicted O/P) using chlorophyll content from heated plants as a predictor. Chlorophyll content was expressed in two different ways: as a percentage of the chlorophyll content in control plants (CHL-C) and as a percentage of the chlorophyll content in heated plants at the beginning of heat treatment (Day 0 of stress treatment) (CHL-HS). The CHL-C and CHL-HS were used as the predictor variable x in Model-C and Model-HS, respectively, to predict O/P. The predictive ability of the model was assessed by comparing prediction root mean square error (RMSPR) to the model root mean square error (RMSE) and reporting the index of agreement (*d* value) (Willmott, 1982; Haboudane et al., 2002; Adomou et al., 2005) and r^2. The RMSPR was calculated as the square root of the mean sums of squares of deviations between predicted values and observed values in the test data. The RMSE was calculated as the standard error of regression for the development data. Because averages of O/P ratios and chlorophyll content were based on three plants for model evaluation, in contrast to five plants from two experiments used in model development, RMSPR was expected to be approximately equal to $\sqrt{3/10}$ RMSE. Also, because prediction errors and model errors are independent, we employed an *F* test to evaluate whether prediction errors in the test data were greater than expected when compared to model error for the development data.

The relationship between the predicted and the measured O/P was compared for each individual genotype separately and also by genotype group. Genotype groups were constructed by combining data from all genotypes within each of three plant groups: hexaploid wheat (6 genotypes), tetraploid wheat (25 genotypes), and maize (20 genotypes).

RESULTS AND DISCUSSION

Model Development

In this study, we developed a model for prediction of thermal damage to photosynthetic membranes, thylakoids.

The prediction is based on estimation of the O/P ratio of chlorophyll *a* fluorescence using chlorophyll content from heat stressed plants as a predictor.

The prediction model for thylakoid damage was developed using 12 cultivars of winter wheat differing in heat tolerance (Ristic et al., 2008). Cultivars were exposed to heat stress, and during exposure to stress treatment, chlorophyll loss and thylakoid damage were assessed. As expected, cultivars were affected by heat stress unequally. Consistent with our previous report (Ristic et al., 2008), heat-susceptible cultivars (Zlatka, Stepa, NS2-4523, Rana Niska, and Kompas) showed greater loss of chlorophyll and greater damage to thylakoid membranes than heat-tolerant cultivars (Proteinka, Ljiljana, Partizanka, NS2-4992, Dragana, Stamena, and Jefimija) (not shown).

We used random coefficients regression models to characterize the relationship between chlorophyll content and O/P and to develop a prediction model for thylakoid damage. For the analysis and model development, chlorophyll content in heat-stressed plants was expressed in two different ways and plotted against O/P (Fig. 1). First, chlorophyll content in heat-stressed plants was expressed as a percentage of the chlorophyll content in control plants (regression analysis C, Fig. 1A). Second, chlorophyll content in heat-stressed plants was expressed as a percentage of the chlorophyll content in the same plants at Day 0 of heat stress (regression analysis HS, Fig. 1B). In both cases, a highly significant negative linear trend ($p < 0.0001$) between chlorophyll content and O/P was observed (Fig. 1) with high coefficient of determination ($r^2 > 0.80$). This confirms our previous report on the linear correlation between chlorophyll content and O/P in winter wheat (Ristic et al., 2007).

Using model development data, fixed effects coefficients from the fitted regression models C and HS yielded equations that constitute the prediction model for O/P (Fig. 1). We refer to these two equations as Model C (Fig. 1A) and Model HS (Fig. 1B):

$$\text{Model C: } Y = -0.0080\ X_C + 0.9947$$

$$\text{Model HS: } Y = -0.0075\ X_{HS} + 0.9469$$

where Y is the predicted O/P ratio of chlorophyll *a* fluorescence in heat stressed plants, X_C is the average chlorophyll content in heat-stressed plants as % of chlorophyll content in control plants, and X_{HS} is the average chlorophyll content in heat-stressed plants as percentage of chlorophyll content in the same plants at Day 0 of heat stress.

The chlorophyll content in heat-stressed plants was expressed in two different ways to test the possibility of using chlorophyll content at the beginning of heat stress as a control. This would be of practical importance for measurements of chlorophyll content and prediction of thylakoid damage under field conditions where environmental factors including temperature are highly variable, making it almost impossible to have control plants that do not experience heat stress.

For model development, chlorophyll content was expressed as a percentage so that the model could apply to natural differences in chlorophyll content among cultivars. Also, environmental factors such as nutrients, for example, show spatial and temporal variation (Farley and Fitter, 1999) that could affect chlorophyll (Minotta and Pinzauti, 1996; Ouzounidou et al., 1997; Haboudane et al., 2002) and thereby contribute error to prediction of O/P. Since the prediction model for O/P relies on chlorophyll, it is crucial that SPAD chlorophyll meter readings are normalized and expressed either as a percentage

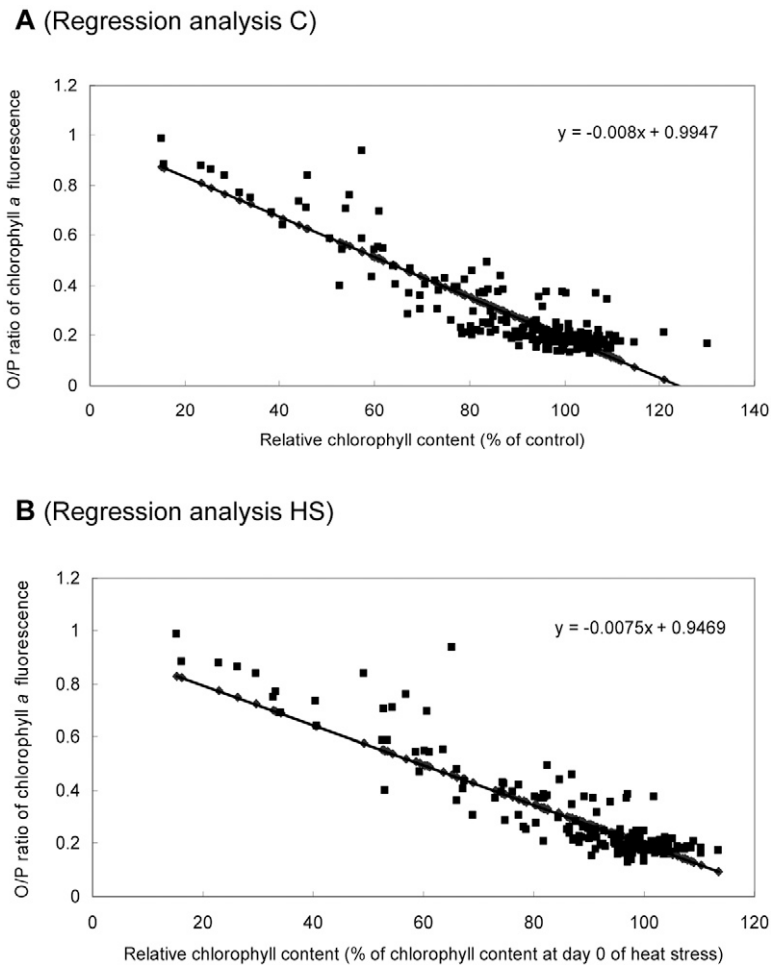

Figure 1. Regression analysis of the relationship between chlorophyll content and O/P, the ratio of constant chlorophyll *a* fluorescence (O) and the peak of variable fluorescence (P), in the flag leaves from 12 winter wheat cultivars experiencing 16 d of heat stress. Chlorophyll *a* fluorescence and chlorophyll content were measured after 0, 2, 4, 6, 8, 10, 12, 14, and 16 d of stress treatment. Data from five replicate plants of each cultivar were averaged, and averages from all 12 cultivars were used for regression analysis. (A) Chlorophyll content from the heat-stressed plants expressed as percentage of the chlorophyll content from control plants. (B) Chlorophyll content from the heat-stressed plants expressed as percentage of the chlorophyll content in the same plants at Day 0 of heat stress.

of chlorophyll content in control plants or as a percentage of chlorophyll content in heat-stressed plants at the beginning of heat stress.

Model Evaluation

Most wheat and all maize genotypes that were used for model testing were affected by heat stress. The effects of heat stress were manifested by loss of chlorophyll and injury to thylakoid membranes (Fig. 2C–F). A few wheat genotypes, however, appeared unaffected by heat, showing no signs of chlorophyll loss and membrane injury (Fig. 2A–B).

We characterized the relationship between chlorophyll loss and heat stability of thylakoid membranes in genotypes of wheat and maize that were used for model testing. A highly significant negative linear correlation ($p < 0.0001$) between chlorophyll content and thylakoid injury was observed in both wheat and maize (Fig. 3). This observation is consistent with our previous report on

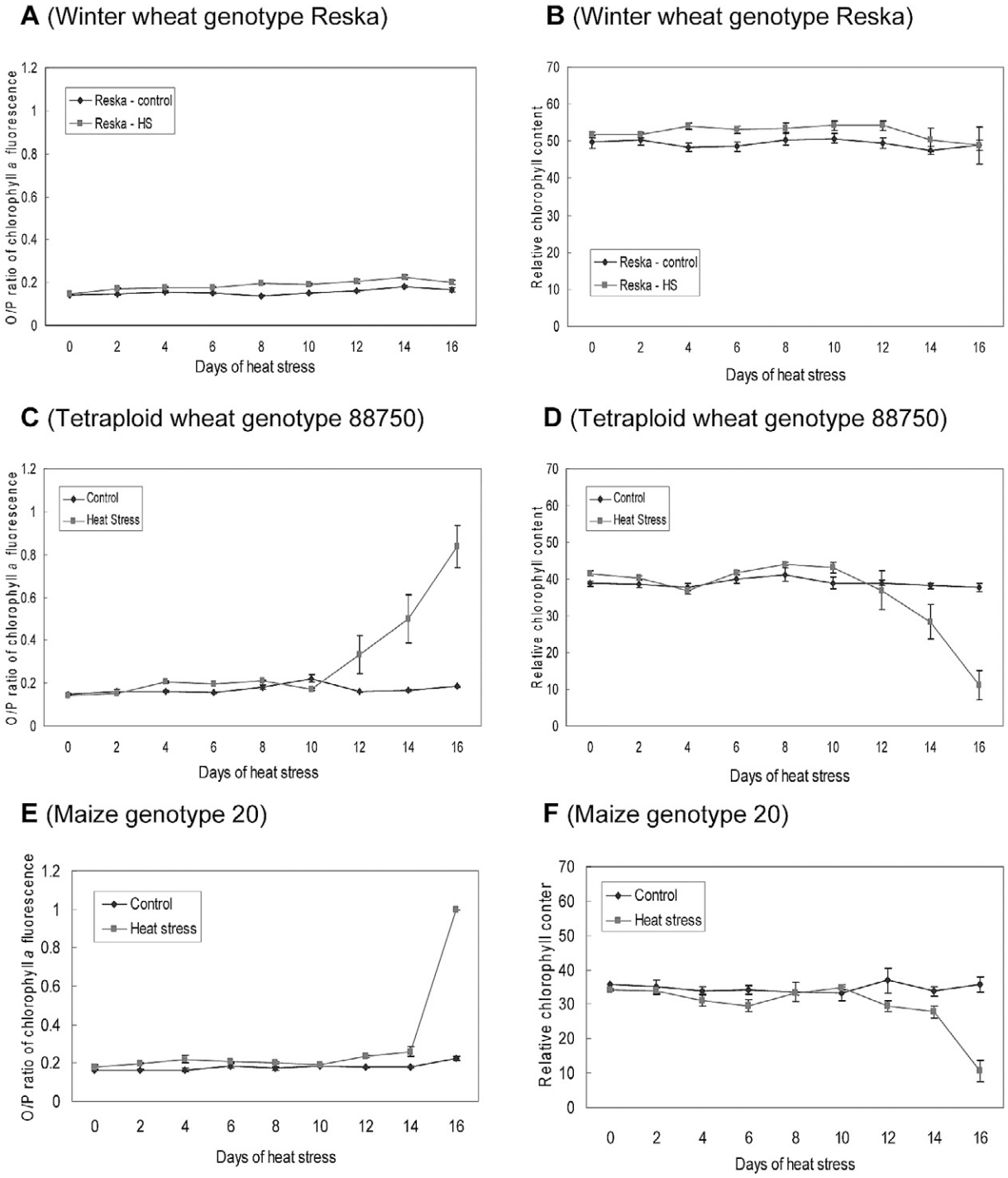

Figure 2. Effect of heat stress on (A, C, E) O/P, the ratio of constant chlorophyll *a* fluorescence (O) and the peak of variable fluorescence (P), and on (B, D, F) chlorophyll content in wheat and maize. Data represent averages of three replicate plants. Bars indicate SEs. Increase in O/P indicates damage to thylakoid membranes; the higher the increase, the greater the damage.

the relationship between chlorophyll content and heat stability of thylakoid membranes in winter wheat (Ristic et al., 2007).

Model C and Model HS predicted O/P of chlorophyll *a* fluorescence in high-temperature–treated plants of wheat and maize. When the predicted and the measured O/P were plotted as a function of time (days of exposure to heat stress), a high similarity between the two variables was obtained (Fig. 4). The similarities between the predicted and the measured O/P values were evident in both the genotypes that were affected by heat stress (Fig. 4C–F) and the genotypes that were not affected by heat stress (Fig. 4A–B).

To evaluate the predictability of the model, we plotted predicted O/P using models derived from the development data against measured O/P in the test data sets. The squared correlation coefficient between observed and predictor (r^2) and index of agreement (*d* value) (Willmott, 1982; Haboudane et al., 2002; Adomou et al., 2005) were also computed and reported. The r^2, however, is indicative of predictive ability of the model only to the extent that it reflects the linear relationship between O/P and chlorophyll content in the test data and has nothing to do with the predictions resulting from the prediction model constructed from the development data. We report r^2 as a measure of predictive potential rather than predictive ability. The *d* values range in value between 0 and 1, with 1.0 indicating perfect agreement between predicted and observed and a value of 0 indicating no agreement. The *d* value is reported because it is more sensitive to systematic error reflected by a prediction model constructed from the development data that is inconsistent with a trend in the test data. Prediction root mean square error was computed and compared to model RMSE, and this comparison was used to test whether prediction errors exceeded models errors used for model development using an *F* test. The model RMSEs were 0.0567 and 0.0551 for models C and HS, respectively.

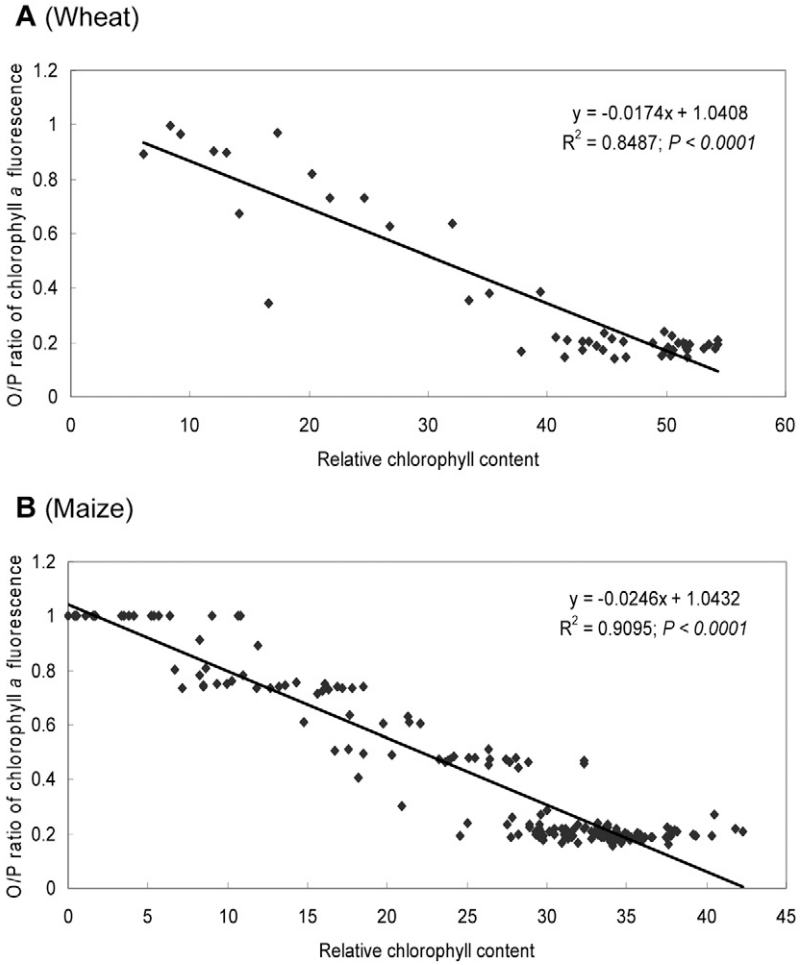

Figure 3. Correlation between chlorophyll content and O/P, the ratio of constant fluorescence (O) and the peak of variable fluorescence (P), in the leaf tissue of heat-stressed plants of (A) wheat and (B) maize used for model testing. Chlorophyll content and chlorophyll *a* fluorescence were measured on Days 0, 2, 4, 6, 8, 10, 12, 14, and 16 of heat-stress treatment. Data represent averages of three replicate plants. (A) Data from three cultivars of winter wheat and three cultivars of spring wheat (n = 51). (B) Data from 20 genotypes of maize (n = 180). Increase in O/P indicates damage to thylakoid membranes; the higher the increase, the greater the damage.

Results for comparing predicted and observed O/P by genotype are listed in Table 1. It should be noted that in wheat genotypes in which chlorophyll content was not affected by heat stress, the prediction model yielded O/P values that were still close to the measured values. In genotype Reska, for example, chlorophyll content was not affected by heat stress (Fig. 2B, Table 1). Yet the RMSPR is reasonably close to what it is expected based on the model RMSE indicating that the prediction model can provide reliable information on the structural and functional state of thylakoid membranes regardless of the effects of heat stress. For model HS, *F* tests revealed significantly ($p < 0.05$) greater prediction error than model error for 3 of the 52 genotypes in the test data, which is approximately the number of significant differences one would expect to find among 52 tests. That is, 0.05(52) = 2.6 tests of the 52 tests are expected to show significance. For Model C, 12 *F* tests revealed significantly greater ($p < 0.05$) prediction error than model error which suggests greater model inaccuracy than for Model HS. Therefore, Model HS rather than Model C is recommended.

For all groups in the groupwise comparisons of predicted and measured O/P, RMSPR and *d* value revealed a good fit and predictive capability of the Model HS (Fig. 5). However, *F* tests revealed prediction error variance to be significantly greater for model error variance for maize for Model C; therefore, only plots of predicted O/P against observed O/P for Model HS are presented in Fig. 5.

The evaluation results strongly suggest that the prediction model developed in our study can for all practical

purposes adequately predict the structural and functional status of thylakoid membranes. The model offers a new approach for quick and inexpensive means of assessing the integrity of photosynthetic membranes in hot environments, thereby providing information on the overall physiological state and heat-stress tolerance in wheat and maize. The model could potentially be used in other crop plants, as the verification of the model indicates that it is not species specific. Furthermore, for assessment of thylakoid damage, control plants may not be necessary as the model can predict O/P using chlorophyll content from heat-stressed plants.

The model for predicting thermal damage to thylakoid membranes relies on chlorophyll content as a predictor. In our study, chlorophyll content was determined with a SPAD chlorophyll meter. Chlorophyll content, however, can also be determined by other noninvasive

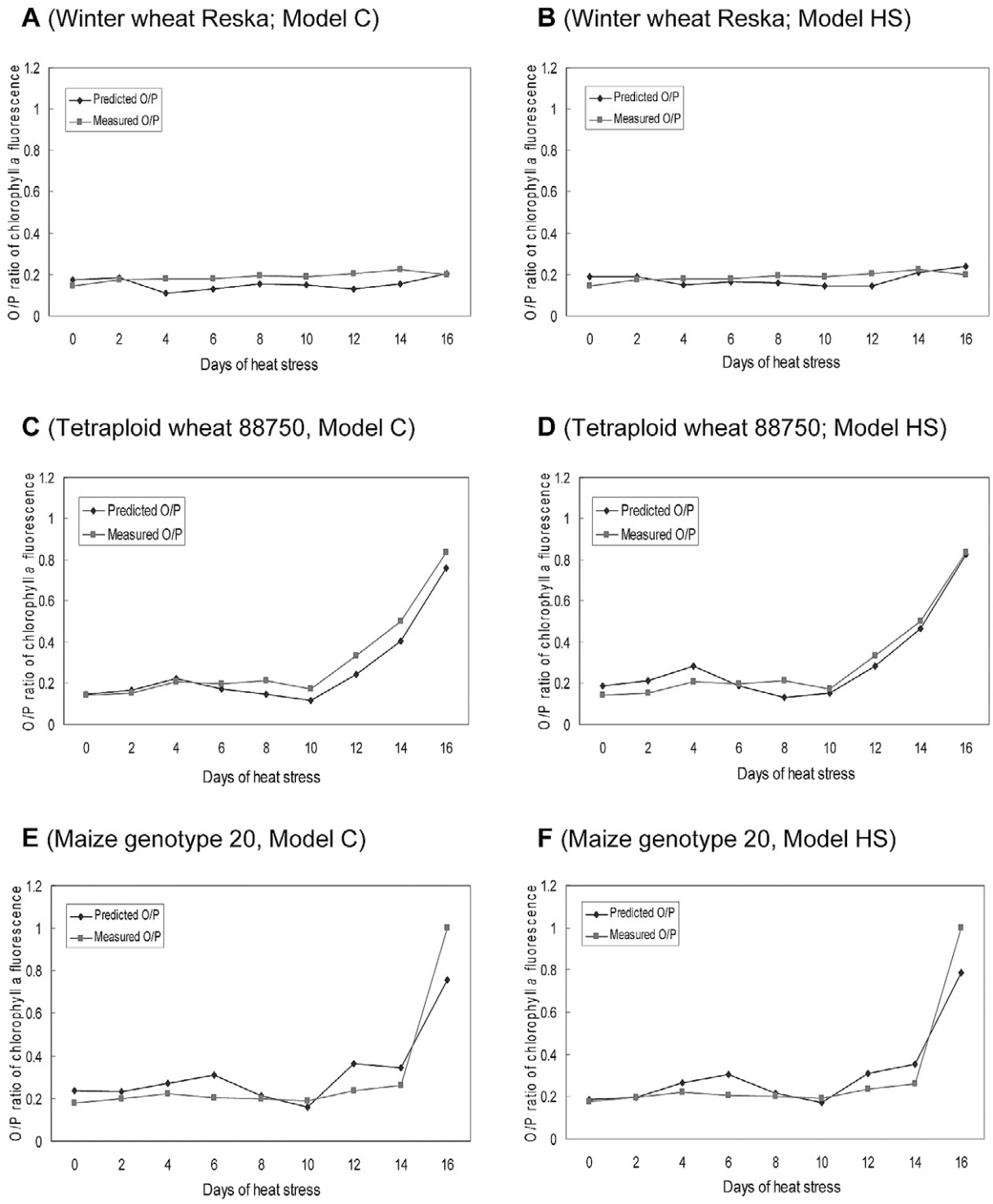

Figure 4. Predicted and measured O/P, the ratio of constant chlorophyll *a* fluorescence (O) and the peak of variable fluorescence (P), in wheat and maize during 16 d of heat treatment. The O/P was predicted using chlorophyll content from heat-stressed plants as a predictor: (A, C, E) O/P predicted using Model C; (B, D, F) O/P predicted using Model HS.

methods, such as remote sensing (Gitelson and Merzlyak, 1997; Haboudane et al., 2002). This raises an interesting possibility of combining the remote sensing data (Gitelson and Merzlyak, 1997; Haboudane et al., 2002) for chlorophyll determination and our model for O/P estimation to detect physiological states of thylakoid membranes and stress tolerance in wheat and maize, and possibly other crop plants.

This model was designed and tested for predicting damage to thylakoid membranes in plants experiencing supra-optimal temperatures. Other abiotic stresses, however, can cause damage to thylakoids (Levitt, 1980), but we do not know if the model can be applied to those situations. If the measurements of chlorophyll content are taken without control plants, it would be important to monitor and control other factors, such as soil water and nutrients, to ensure that the plants are not affected by stresses other than high temperature. Furthermore, our model was tested on plants at their advanced growth stage, flowering in wheat and preflowering in maize. This was done because, most commonly, wheat and maize experience heat stress during flowering and postflowering stages. Hence, in most cases, the measurements of chlorophyll content under heat-stress conditions could begin shortly before flowering or at the beginning of flowering and continue thereafter for 7 to 21 d (Ristic et al., 2007). Heat-induced chlorophyll loss and thylakoid damage will probably depend on environmental conditions and plant heat tolerance. If air temperature is not sufficiently high or the high temperature does not last for a prolonged period of time, detectable loss of chlorophyll may not occur. Therefore, if the model is used for assessing plant heat tolerance, it is important that measurements of chlorophyll content are taken in environments experiencing sufficiently high supra-optimal temperatures.

In summary, in this study, we developed a model for prediction of heat stability of thylakoid membranes. The prediction is based on estimation of the O/P ratio of chlorophyll *a* fluorescence using chlorophyll content as a predictor. The model showed a good prediction of O/P in both wheat and maize. This model could be used as an easy means for detection of physiological states and tolerance to heat stress in wheat and maize.

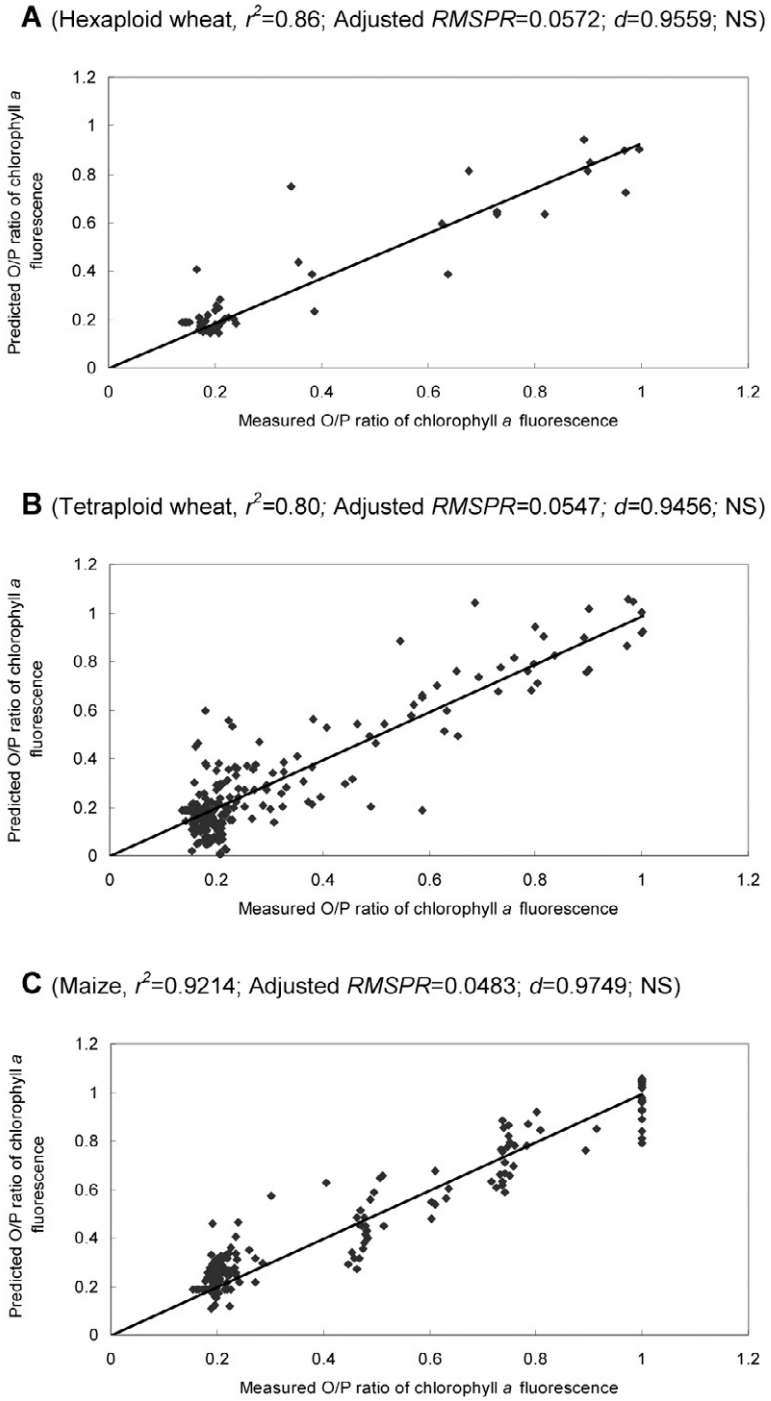

Figure 5. Comparison between measured O/P, the ratio of constant chlorophyll *a* fluorescence (O) and the peak of variable fluorescence (P), and O/P values predicted by Model HS. (A) Data from six genotypes of hexaploid wheat (three genotypes of winter wheat and three genotypes of spring wheat). (B) Data from 25 genotypes of tetraploid wheat. (C) Data from 20 genotypes of maize. NS, not significant.

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